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Clinical and Epidemiological Implications of Swine Hepatitis E Virus Infection

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In nonendemic areas, most patients with acute hepatitis E were infected through traveling to endemic areas. However, some patients did not have a history of foreign travel before infection. Furthermore, high seroprevalence rates of antibody to hepatitis E virus (anti-HEV) were found in the general adult population in some countries without any recorded outbreak of hepatitis E. The significance of anti-HEV assay in these subjects remains obscure. To study if swine might be a source of HEV infection, HEV was tested in sera of 235 pigs in Taiwan, and from 5 patients with acute HEV infection who either denied or did not provide any foreign travel history. Three (1.3%) pigs had detectable swine HEV RNA. The swine and human HEV strains from Taiwan formed a monophyletic group, distinct from three previously reported groups: the United States human and swine HEV strains, the Mexico strain, and the largest group composed of the Asian and the African strains. The identity of nucleotide sequences was 84–95% between swine and human HEV strains in Taiwan, and 72–79% between Taiwan strains and those from different areas. The predicted amino acid sequence of a Taiwan swine HEV strain within the peptide 3-2 used in commercial anti-HEV assay showed a high identity (91–94%) with those of other human and swine HEV strains. Swine may be a reservoir of HEV and subclinical swine HEV infection may occur. Cross-reactivity of current anti-HEV assay may account for the high prevalence rate of anti-HEV in the general population in nonendemic areas. ***J. Med. Virol. 60:166–171, 2000.***

KEY WORDS: phylogenetic analysis; polymerase chain reaction; swine hepatitis E virus; viral hepatitis

INTRODUCTION

Hepatitis E virus (HEV) is usually transmitted by fecal contamination of drinking water [Balayan et al., 1983; Velazquez et al., 1990; Tsega et al., 1991; Hyams et al., 1992; Skidmore et al., 1992; Krawczynski, 1993; Nanda et al., 1994]. HEV infection is more prevalent in developing countries in tropical and subtropical areas [Wong et al., 1980; Velazquez et al., 1990; Tsega et al., 1991; Aye et al., 1992; Favorov et al., 1992; Hyams et al., 1992; Skidmore et al., 1992; Huang et al., 1995]. In western countries, it is usually considered to be an "imported disease" by traveling, visiting, trading, or immigration [De Cock et al., 1987; Mast et al., 1997]. The survey and diagnosis of HEV infection are usually based on the detection of antibody to HEV (anti-HEV) [Tsega et al., 1991; Favorov et al., 1992; Hyams et al., 1992; Skidmore et al., 1992; Nanda et al., 1994]. Anti-HEV assay is of diagnostic value in endemic areas as shown by detection rates of 71–100% in outbreak cases. However, a high seroprevalence rate of anti-HEV was also found in general adult populations in some countries without any recorded outbreak of hepatitis E [Lok et al., 1992; Lee et al., 1994; Mast et al., 1997; Thomas et al., 1997]. Many of these anti-HEV-positive subjects did not have a history of hepatitis or foreign travel, and most were not verified further by detection of HEV RNA. The significance of the positive anti-HEV testing in these subjects is unclear.

Documented HEV infection by detecting and sequencing of viral RNA has been reported recently in Taiwan [Wu et al., 1998]. Most of these patients with acute hepatitis E had a foreign travel history to an endemic area within 3 months of acute hepatitis. Phy-

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logenetic analysis of HEV genomes from these subjects suggested that the sources of infection may have originated from endemic areas where they had traveled before the development of hepatitis. Nevertheless, a few anti-HEV-positive cases with acute hepatitis did not have any history of foreign travel. The origin of their infection or the significance of the anti-HEV in these subjects remained unknown [Thomas et al., 1997; Wu et al., 1998].

Recently, a swine HEV with many similarities in both nucleotide and amino acid sequences to a human HEV strain from United States was cloned [Meng et al., 1997; Schlauder et al., 1998]. This study was designed to study the prevalence of swine HEV viremia in pigs from herds at different areas of Taiwan. Swine HEV was cloned and sequenced to determine the identity in nucleotide and amino acid sequences between the swine and the human HEV strains, particularly those from patients without travel history.

MATERIALS AND METHODS

Swine and Human Serum Samples

A total of 235 serum samples were collected randomly from herds at different areas (20 pigs from the northern area, 53 from the middle area, 72 from the southern area, and 90 from the eastern area) of Taiwan. Of these pigs, 191 were 2–5 months old and the remaining 44 were 6–7 months old. Pigs younger than 2 months were still breast fed, and those older than 6 months were ready for sale. From May 1990 to October 1998, 14 patients (12 men and 2 women; aged 23–68 years old) with documented acute hepatitis E and detectable HEV RNA were assessed at the Veterans General Hospital-Taipei [Wu et al., 1998]. Of these, 11 patients had a recorded travel history, with 9 (82%) having traveled to endemic areas (China 7, South East Asia 1, Mexico 1) within 3 months before the onset of acute hepatitis; 2 denied such a history. The travel history in the remaining 3 cases had not been recorded. In a previous study [Wu et al., 1998], HEV genomes from those patients with a travel history to China were shown to be closely related to the Guangzhou strains by phylogenetic analysis. To study if there was any novel strain, the serum samples from the two patients without any travel history and the three patients without recorded travel history were analyzed in this study. The tests in these five patients were negative for serum immunoglobulin M antibody to hepatitis A virus (IgM anti-HAV), hepatitis B surface antigen (HBsAg), IgM antibody to hepatitis B core antigen (IgM anti-HBc), and antibody to hepatitis C virus (anti-HCV). None of these five patients had been involved in work dealing with pigs. The human and swine serum samples were stored at –70°C until used for cloning of HEV genomic sequences.

Detection, Cloning, Sequencing, and Phylogenetic Analysis of HEV RNA

Reverse transcription-polymerase chain reaction (RT-PCR) was carried out as described previously [Wu et al., 1998]. Two sets of primers (the first set: external primers 3,156 and 3,157, internal primers 3,158 and 3,159; the second set: external primers 3,160 and 3,161, internal primers 3,162 and 3,163) were synthesized and used in nested PCR as reported previously [Meng et al., 1997]. Briefly, viral RNA templates were extracted from 50 μl of serum and were reverse transcribed. Nested PCR was then carried out by using the outer and inner primers, accordingly, for 35 cycles each round. Strict procedures were followed to avoid false-positive results [Kwok and Higuchi, 1989]. To avoid carry over, all tips used in the experiments had filters and were disposed of at every step of the experiments. All the buffers, reagents, and primers were separated into aliquots that were discarded after each experiment. The experiments were repeated for each case. Consistent positive or negative results were obtained. To avoid contamination, cloning was undertaken separately for each positive sample. The amplified PCR products were ligated into the plasmid pCR2 vector (Original TA Cloning Kit, Invitrogen, La Jolla, CA) according to the manufacturer's instructions. The ligation mixture was used to transform the competent *Escherichia coli* strain DH5α (Gibco BRL, Life Technologies, Gaithersburg, MD) [Chung and Miller, 1988; Wu et al., 1998]. Multiple (>8) positive colonies were picked up and cultured in LB-broth. Plasmid DNA was extracted and subjected to the dye terminator cycle sequencing reaction according to the standard protocol provided by the manufacturer (Dye terminator cycle sequencing core kit #402117, Perkin Elmer, Norwalk, CT). The sequencing products were precipitated with alcohol and analyzed in an ABI 373A sequencer (Perkin Elmer). HEV sequences were aligned by multiple alignments using the CLUSTAL V Program by Higgins et al. [1992] Phylogenetic analysis by the neighbor-joining method using the Molecular Evolutionary Genetics Analysis Program (MEGA, Version 1.01, Kumar et al. [1993]) was used for the phylogenetic analysis of sequencing data [Swofford et al., 1996]. Neighbor-joining analyses were conducted by calculating the Kimura's 2-parameter distances [Kimura, 1980]. The confidence of the clades, i.e., the monophyly, was tested by bootstrapping (a resampling statistical technique) with 1,000 replicates of heuristic searches. The nodes with bootstrap values greater than 70% are significantly supported with 95% confidence (robustness) [Hillis and Bull, 1993].

Serological Markers

The following viral markers were examined by radioimmunoassay kits: IgM anti-HAV, HBsAg and IgM anti-HBc (HAVABM, Ausria II-125 and CORAB-M; Abbott Laboratories, North Chicago, IL). Anti-HCV was tested by a second-generation enzyme immunoassay (Abbott Laboratories). Anti-HEV was measured by a solid-phase enzyme-linked immunosorbent assay (ELISA; Genelabs and Diagnostic Technology, Singapore) as reported previously [Thomas et al., 1997; Wu et al., 1998].

TABLE I. Detection of Swine HEV RNA in Different Areas of Taiwan*

Area	No. of pigs	No. with HEV RNA (%)
Northern	20	0 (0)
Middle	53	1 (1.9%)
Southern	72	1 (1.4%)
Eastern	90	1 (1.1%)
Total	235	3 (1.3%)

*HEV, hepatitis E virus.

RESULTS

Prevalence of HEV RNA in Swine

As shown in Table I, 3 (1.3%) of the 235 pigs had detectable swine HEV RNA. These 3 pigs were from the middle, southern, and eastern areas, respectively. Two positive samples were detected using the first set of primers (external: 3,156 and 3,157, internal: 3,158 and 3,159), and the third was detected by using the second set of primers (external: 3,160 and 3,161, internal: 3,162 and 3,163). The ages of the 3 pigs were 2, 3, and 5 months, respectively. None of the 44 pigs older than 5 months had swine HEV RNA.

Cloning, Sequencing, and Phylogenetic Analysis of Swine and Human HEV

The PCR products generated from the swine and the human HEV genomes were cloned and sequenced. Multiple clones from each human or swine serum sample were sequenced. The identity in nucleotide sequences between different clones from a single serum sample ranged from 99.3% to 100%. Actually, it was 100% identical among most clones. Phylogenetic analysis was performed based on the sequence of PCR products from nt 5,972 to 6,319 (Fig. 1). GenBank accession numbers of the following HEV isolates are indicated in parentheses. Surprisingly, the sequence of a swine HEV strain, TW32SW (Genbank accession number, AF117280), was very close to that of a human HEV strain, TW5483E (Genbank accession number, AF117277). These two isolates joined the other swine HEV strain, TW74SW (Genbank accession number, AF117281), to form a subgroup which was supported by a bootstrap value of 99%. Four HEV isolates from the patients, TW6310E (Genbank accession number, AF117279), TW8E (Genbank accession number, AF117275), TW6196E (Genbank accession number, AF117278), and TW2494E (Genbank accession number, AF117276) formed the other monophyletic subgroup supported by a bootstrap value of 98%. These two subgroups subsequently formed a major monophyletic group, significantly different from the remaining human or swine HEV isolates. The human HEV strain Hevus2 (AF035437) and the swine HEV strain (AF011921) from the United States formed the second major group supported by a bootstrap value of 100% [Meng et al., 1997; Schlauder et al., 1998]. Hpenssp (M74506) from Mexico appeared to form independently the third group [Huang et al., 1992]. The human HEV strains (AF020603 to AF020608) from Nepal; Heu 22532 (U22532) and Hevorfs (X99441) from India; Hpesvp (M73218) from Burma [Tam et al., 1991]; and Hpeppg (D90274) and Hpehev (D10330) from Myanmar formed a monophyletic subgroup supported by a bootstrap value of 90%. Hpegensa (L08816), Hpecg (D11092) [Aye et al., 1992], Hpeuigh (D11093), Hpegena (L25547), and Hpeorfs (L25595) from Xingjiang; Hpcegenom (M94177) from HeBei; and Hpea (80581) from Pakistan [Tsarev et al., 1992] formed a small monophyletic subgroup supported by a bootstrap value of 100%. The latter two subgroups, the Hevcg (X98292) from India and the Heu62121 (U62121) from Chad, subsequently formed the largest fourth major group as shown in Figure 1.

Comparison of Nucleotide and Amino Acid Sequence Between Human and Swine HEV

As shown in Table II, identity in nucleotide sequences from nt 5,972 to 6,319 between the swine and the human HEV strains in Taiwan ranged from 84% to 95%. The identity in nucleotide sequence between Taiwan and US HEV strains ranged from 75% to 79%. The identity in nucleotide sequence between Taiwan and the Mexico HEV strains ranged from 72% to 73%. And the identity in nucleotide sequence between Taiwan and the fourth group ranged from 75% to 77%.

The anti-HEV assay available commercially (Genelabs and Diagnostic Technology) is composed of the carboxyl terminal 33 amino acids of the open reading frame (ORF) 3 of the HEV (4-2 of the Mexico strain [Huang et al., 1992]; 6-1-4 of the Burma strain [Tam et al., 1991]) and the carboxyl terminal 42 amino acids of the ORF2 of the HEV (3-2, the Mexico and the Burma strains) [Tam et al., 1991; Huang et al., 1992; Yarbough et al., 1991]. The predicted amino acids within the peptide 3-2 of a swine HEV strain in Taiwan, TW23SW, showed a very high identity (91–94%) with those of other human HEV isolates or the swine HEV strain from the United States (Af011921).

DISCUSSION

In this study, swine HEV RNA was detected in 1.1–1.9% of pigs from different herds, indicating that swine HEV infection appeared to be prevalent in different areas of Taiwan. The failure to detect HEV RNA in pigs from herds located in the northern part of Taiwan might be due to the small number of pigs tested. Because HEV viremia is transient [Krawczynski, 1993; Meng et al., 1997; Wu et al., 1998], the detection rate of HEV viremia probably underestimated the prevalence of HEV infection in pigs. However, the results of this study indicate clearly that a few pigs were potentially infectious and might be infectious to other pigs at the time of screening. In support of this view, Meng et al. [1997] showed that the prevalence of swine anti-HEV increased with age, and all pigs had been infected during the adult age. All HEV RNA-positive pigs in this study were younger than 6 months, and those older than 6 months might have been already infected and cleared the virus. An assay is being developed for the

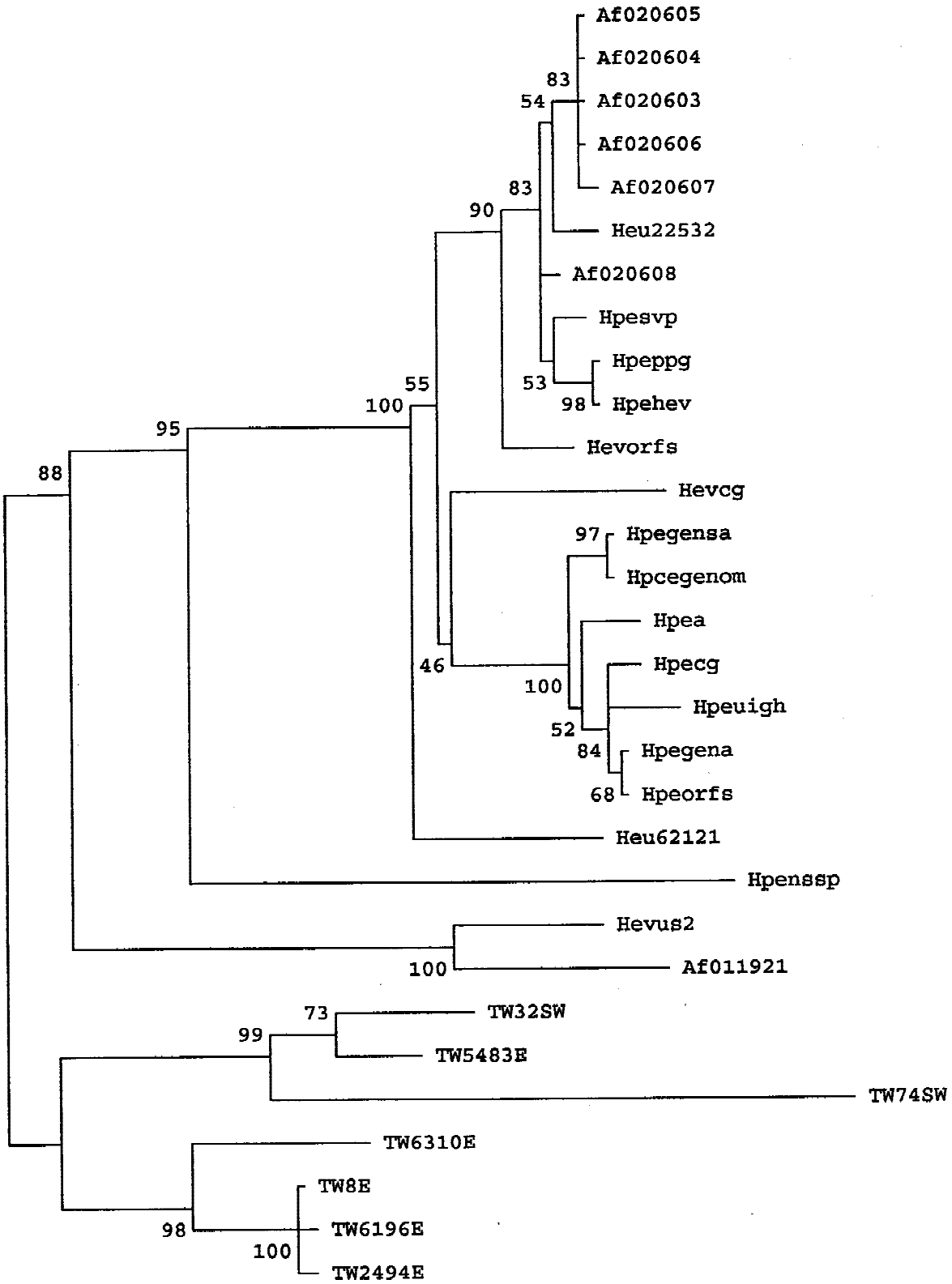

Fig. 1. Phylogenetic analysis of swine and human hepatitis E virus (HEV). Molecular Evolutionary Genetics Analysis Program (MEGA, Version 1.01, Kumar et al. [1993]) was used. The number on each node indicates a bootstrap value (shown by percentage). The nodes with bootstrap values > 70% are significantly supported with ≧95% confidence. The GenBank accession numbers of HEV isolates are indicated in parentheses and sources of these isolates are shown in the following. TW32SW, TW74SW, TW5483E, TW6310E, TW8E, TW6196E and TW2494E were cloned in our laboratory. TW: Taiwan, SW: swine. Human HEV strain Hevus2 (AF035437) and a swine HEV strain (AF011921) from the United States; Hpenssp (M74506) from Mexico; (AF020603 to AF020608) from Nepal; Heu 22532 (U22532), Hevorfs (X99441) and Hevcg (X98292) from India; Hpesvp (M73218) from Burma; Hpeppg (D90274) and Hpehev (D10330) from Myanmar; Hpegensa (L08816), Hpecg (D11092), Hpeuigh (D11093), Hpegena (L25547) and Hpeorfs (L25595) from Xingjiang; Hpcegenom (M94177) from HeBei, Hpea (80581) from Pakistan; Heu62121 (U62121) from Chad.

TABLE II. Identity (%) Between Human and Swine HEV Nucleotide Sequences*

Strains	Source	Areas	TW32SW	TW74SW	TW5483E	TW8E	TW6310E
TS32SW	Swine	Taiwan	100	91	95	85	84
TW74SW	Swine	Taiwan	91	100	93	84	86
TW5483E	Human	Taiwan	95	93	100	85	84
TW8E	Human	Taiwan	85	84	85	100	92
TW6310E	Human	Taiwan	84	86	84	92	100
AF020607	Human	Nepal	77	76	77	79	78
Hpesvp	Human	Burma	76	75	76	78	77
Hpehev	Human	Myanmar	76	76	77	79	78
Hevorfs	Human	India	75	75	76	79	77
Hevcg	Human	India	77	76	75	75	77
Hpegensa	Human	Xingjiang	76	76	76	78	77
Hpea	Human	Pakistan	77	77	77	78	77
Hpegena	Human	Xingjiang	76	76	77	78	77
Heu62121	Human	Chad	75	76	75	79	77
Hpenssp	Human	Mexico	72	73	73	77	75
Hevus2	Human	USA	76	78	76	76	78
AF011921	Swine	USA	75	77	79	75	76

*HEV, hepatitis E virus

detection of swine anti-HEV so that the actual prevalence of swine HEV infection in this area will then be determined.

Consistent with previous reports [Meng et al., 1997; Schlauder et al., 1998], this study also showed four major groups (or genotypes) of HEV by phylogenetic analysis. It appeared that the grouping of HEV isolates was closely related to geographic distribution. In a previous study [Wu et al., 1998], human HEV strains (TW4E, 7E, and 8E) from Taiwan formed a major monophyletic group with the isolates from Guangzhou [Huang et al., 1995] by phylogenetic analysis based on ORF1 sequence of HEV. In this study, the same TW8E and other human and swine HEV isolates from Taiwan also formed a major monophyletic group by phylogenetic analysis based on a different segment (ORF2) of HEV sequence. Although the ORF2 sequences of the Guangzhou strains were not available for phylogenetic analysis, it is reasonable to assume that the Guangzhou strains and all currently isolated human and swine HEV strains from Taiwan belong to the same genotype. The newly discovered human and swine HEV strains from the United States belong to another distinct genotype. The Mexico strain belongs to a separate and unique monophyletic group and is highly divergent in nucleotide sequences from other HEV genotypes. The largest major group is composed of the Nepal, Burma, India, Pakistan, HeBei, and Xingjiang strains. Although the Chad strain is different from many strains of the largest major group, it still belongs to this group according to phylogenetic analysis.

HEV has been detected in domestic pigs [Balayan et al., 1990; Clayson et al., 1995]. Currently, only one swine HEV strain from the United States has been isolated and sequenced [Meng et al., 1997]. The present study isolated three swine HEV strains from different areas of Taiwan. Although swine HEV infection was known to exist in Taiwan, the identity of nucleotide sequences between Taiwan and US swine strains was only 75–77%, lower than the identity (84–95%) between human and swine HEV strains from Taiwan. It will be interesting to determine whether the high degree of identity in nucleotide sequences between the human and the swine HEV strains also exists in other areas.

Consistent with previous studies [Meng et al., 1997; Schlauder et al., 1998], high identity in nucleotide sequences between human and swine HEV strains in Taiwan was found. This finding suggests that cross-species infection is highly likely, and swine may be a reservoir of HEV. Meng et al. [1998] reported genetic and experimental evidence for cross-species infection by swine HEV in rhesus monkeys and a chimpanzee. In a reciprocal experiment, specific-pathogen-free pigs were infected with the US-2 strain of human HEV [Meng et al., 1998]. It appeared that cross-species infection usually resulted in subclinical infection with minimal liver histological change [Meng et al., 1998]. The possibility of development of clinical hepatitis caused by swine HEV infection in humans on some occasions could not be excluded. The very high identity in amino acid sequence over type-common epitopes used in the anti-HEV assay raises a concern of possible cross-reactivity in clinical diagnosis and epidemiological surveys [Yarbough et al., 1991]. In the report by Meng et al. [1997], swine anti-HEV cross-reacted with human HEV capsid antigen. If humans are infected by swine HEV or exposed to its antigen, cross-reactivity in the anti-HEV assay may occur. This may explain the high prevalence of anti-HEV in the general population from nonendemic areas who did not have a history of hepatitis or foreign travel [Lok et al., 1992; Lee et al., 1994; Mast et al., 1997; Thomas et al., 1997]. A more specific assay is needed to differentiate between human and swine HEV infections. Raising and selling pigs are common and pork is the main source of meat in Taiwan. However, there has been no outbreak of HEV infection in Taiwan. There are several possible explanations: firstly, general hygienic conditions in Taiwan have been improved greatly in recent years, which reduces markedly the chance of contamination of drinking water by swine sewage; secondly, HEV infection is

self-limited and occurs at an early age of pigs before slaughter; thirdly, pork is usually well-cooked before consumption; and fourthly, cross-species infection may occur subclinically and thus may be undiagnosed.

In a previous report [Wu et al., 1998], most patients with HEV infection revealed a travel history to endemic areas within 3 months before the onset of acute hepatitis. Phylogenetic analysis of HEV genomes also indicated that the source of infection originated from the endemic areas where the patients had visited before experiencing acute hepatitis. Nevertheless, a minority of the patients (such as TW5483E and TW2494E) denied a travel history to endemic areas before hepatitis. The HEV genomic sequences of these patients are closer to the swine HEV strain of the same area than other human HEV strains. Although these patients were not involved in dealing directly with pigs, the possibility of cross-species swine HEV infection could not be excluded. Alternatively, there might be other animal species that could also be reservoirs of HEV [Maneerat et al., 1996].

Xenotransplantation may be of clinical value in the future because of the shortage of available human donor organs. Screening donor pigs for swine HEV may be important. The findings of the present study indicate that swine HEV infection is prevalent in pigs from Taiwan, and swine HEV is genetically and in its evolution close to human HEV. Swine HEV may cross-infect and become pathogenic in humans under immunocompromised conditions after transplantation.

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